REMARKS

1. Claim Amendments

Claim 1 has been amended to incorporate the feature that the method is performed *in vitro*. Claim 1 has been further amended to incorporate the feature that the mismatch repair gene is a *PMS2* gene. Claim 1 has also been amended to link the final step to the preamble such that the method is for making a mutation in the gene of interest and stabilizing the genome of the cell. The amendments were suggested by the Examiner and are supported throughout the specification. No new matter is added.

Claims 6 and 7 have been amended to improve clarity. No new matter is added.

2. 35 U.S.C. §112, first paragraph (Enablement)

The Office Action rejects claims 1-7 under 35 U.S.C. §112, first paragraph, alleging that the claims are not enabled by the specification. The Office Action states that the specification is enabling "for a method to generate a mutation in a mammalian cell in vitro comprising growing a hypermutable mammalian cell comprising the gene of interest and a polynucleotide comprising PMS2134." (Paper 18, page 4, lines 8-10.) However, the Office Action alleges that the specification does not provide sufficient enablement for use of any dominant-negative mutant of *PMS2* or of any mismatch repair gene. Applicants respectfully traverse.

The amended claims are directed to an *in vitro* method for generating a mutation in a gene of interest in a hypermutable cell and subsequently stabilizing the genome of the cell. A hypermutable mammalian cell comprising the gene of interest and a dominant-negative allele of a *PMS2* mismatch repair gene under control of an inducible

transcriptional regulatory element is grown. The cell is tested to determine whether the gene of interest harbors a mutation. Mismatch repair activity is restored to the cell by decreasing expression of the dominant-negative allele, thereby stabilizing the genome of the cell.

The specification and knowledge of the skilled artisan at the time the application was filed demonstrate that one of skill in the art could have made and used the dominant-negative *PMS2* genes recited in the claims. The specification describes a human PMS2 truncated mutant protein that confers a dominant-negative effect on mismatch repair. The specification also discloses a homologous mouse PMS2 protein (SEQ ID NO:6). Overall, the human and mouse PMS2 sequences are 73% identical. The N-terminal 134 amino acid residues are highly conserved and share 89.5% sequence identity. (Exhibit A.) As expected from the level of homology, the mouse PMS2 functions in the same manner as the human protein. Narayanan teaches that "mice nullizygous for *Pms2* showed a 100-fold elevation in mutation frequency in all tissues examined compared with both wild-type and heterozygous litter mates." (Page 3122, lines 8-11 of the Abstract; *Proc. Natl. Acad. Sci.* (1997) *94*:3122-3127; Exhibit B.) A plant PMS2 protein that was also known at the time of filing further demonstrates that PMS2 proteins are structurally and functionally similar. As shown in the attached Declaration of Dr. Nicholas C. Nicolaides, an *Arabidopsis thaliana* PMS2 was designed with a similar truncation mutation (AtPMS134). (Exhibit C; see Fig. 1.) The AtPMS134 protein exerted a similar dominant-negative effect in bacteria. (Exhibit C; see Figure 2.)

The species source of the dominant-negative alleles of *PMS2* mismatch repair genes in these examples are highly divergent (plant, mouse and human PMS2). One of

skill in the art would therefore have no reason to doubt that a truncated PMS2 from other species would have a dominant-negative effect on mammalian mismatch repair.

One of skill in the art would also be able to identify other mutations in the *PMS2* gene that are dominant-negative. Pang was published before the effective filing date of the application. (*Mol. Cell. Biol.* (1997) *17*:4465-4473; Exhibit D.) Pang teaches several PMS2 proteins that exert a dominant-negative phenotype in yeast, mutant proteins Pms1p(692-904) and Pms1p-F126A, at Table 1 on page 4470. The yeast homolog of PMS2 is PMS1P. Thus Pang teaches a truncated and a mutant PMS2 protein that cause a dominant-negative mismatch repair phenotype in yeast. Pang demonstrates that it would not require undue experimentation to make other PMS2 mutations that confer a dominant-negative mismatch repair phenotype in a cell.

Further, the Applicants teach routine biological (β-galactosidase) assays that can be used to determine if a *PMS2* mutation has the dominant-negative effect on mismatch repair. (Specification, Example 1.) The assays provided in the specification are reproducible, routine procedures. Thus, the experimentation needed to identify a dominant-negative *PMS2* gene is not undue. One of ordinary skill in the art could readily determine mutations (particularly truncation mutations) that result in a dominant-negative phenotype.

Applicants submit that the claims as amended are fully enabled by the specification.

3. 35 U.S.C. §112, first paragraph (Written Description)

The Office Action rejects claims 1-7 under 35 U.S.C. §112, first paragraph as allegedly lacking an adequate written description.

Applicants respectfully request reconsideration of this rejection in view of the statutory basis of the Written Description Requirement and the current case law as developed by the United States Court of Appeals for the Federal Circuit.

In a recent Federal Circuit decision, *Moba, B.V., Staalkat, B.V., and FPS Food Processing Systems, Inc. v. Diamond Automation, Inc.* 2003 U.S. App. LEXIS 6285 (Fed. Cir. 2003), the Federal Circuit discussed the written description requirement at length. The Federal Circuit explained that its own case law shows two primary goals in the written description requirement. The first is embodied in its decision in *In re Wertheim* 541 F.2d 257, 191 USPQ 90 (CCPA 1976), and the second is embodied in its decision *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). The *Wertheim* court noted that "the function of the description requirement is to ensure that the inventor had possession, as of the filing date of the application relied on, of the specific subject matter *later* claimed by him." *Wertheim* at 541 F.2d 257, 262, 191 USPQ 90, 96. As restated more recently by the Federal Circuit:

> The purpose of the written description requirement is to prevent an applicant from *later* claiming that he invented that which he did not; the applicant for a patent is therefore required "to recount his invention in such detail that his *future claims can be determined to be encompassed within his original creation.*"

Amgen Inc. v. Hoechst Merion Roussel Inc., 314 F.3d 1313, 1330, 65 USPQ2d 1385, 1397 (Fed. Cir. 2003) (citing *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1561, 19 USPQ2d 1111, 1115 (Fed. Cir. 1991) (emphasis added)).

The second goal of the written description requirement was addressed in *Regents of the University of California v. Eli Lilly & Co.* The *Eli Lilly* court applied the written description requirement to adequacy of a description of a DNA sequence. The court held that a precise definition of the DNA sequence was required to satisfy the written description requirement, even in the absence of priority issues. The court has further refined this rule in such cases as *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 296 F.3d 1316, 63 USPQ2d 1069 (Fed. Cir. 2002) and *Amgen Inc. v. Hoechst Merion Roussel Inc.*, 314 F.3d 1313, 65 USPQ2d 1385 (Fed. Cir. 2003). In *Amgen*, the Federal Circuit clarified its holding in *Eli Lilly*, stating: "*Eli Lilly* did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular known structure." *Amgen Inc. v. Hoechst Merion Roussel Inc.*, 314 F.3d at 1332. Moreover, a representative number of species within a claimed genus may fulfill the written description requirement. *The Regents of the University of California v. Eli Lilly and Company* 119 F.3d 1559, 1568 (Fed. Cir. 1997).

The amended claims do not go beyond the scope of the originally filed claims. Thus the amended claims satisfy the first goal of the written description requirement. Clearly, the Applicants were in possession of the claimed invention at the time of filing the application.

Further, the Applicants have satisfied the second goal of the written description requirement by showing that "the disclosed function is sufficiently correlated to a known, particular structure." The Applicants demonstrated that truncation mutants of PMS2

proteins exert a dominant-negative effect. Applicants demonstrated by working example that a dominant-negative form of the PMS2 protein, PMS2-134, exerts a dominant-negative effect on cells. Thus a known structure is associated with a known function. In addition, the applicants describe mouse PMS2 protein (SEQ ID NO: 6) which is homologous to the human PMS2. The function of the mouse PMS2 protein was also known at the time the application was filed. Thus the amino acid sequence and function of PMS2 proteins were known to be correlated. The Declaration of Dr. Nicolaides is further evidence that the PMS2 protein of a diverse species has a similar function and structure. (Exhibit C) The declaration states that a truncated *A. thaliana* PMS2 functions as a dominant-negative inhibitor of mismatch repair. Thus the structure and function are again shown to correlate.

Applicants submit that the specification fulfills the Written Description Requirement with respect to the amended claims.

4. 35 U.S.C. §112, second paragraph

Claim 1 has been amended to link the final step to the preamble. It is believed that this amendment renders the claim sufficiently definite to overcome the rejection under 35 U.S.C. §112, second paragraph. Applicants respectfully request withdrawal of the rejection.

5. 35 U.S.C. §103(a)

The Office Action rejects claims 1-7 under 35 U.S.C. §103(a), as obvious over Nicolaides *et al.* (1998) *Mol. Cell. Biol.* 18:1635-1641 ("Nicolaides I"), or U.S. Patent

No. 6,146,894 to Nicolaides *et al.* ("Nicolaides II") in view of PCT Publication No. WO 96/01313 to Bujard *et al.* ("Bujard").

Nicolaides I is cited as teaching stably transfected cell lines that express a truncated human PMS2 protein having only amino acid residues 1-133. (Paper 18, page 7, lines 7-9.) Nicolaides II is cited as teaching a method of producing a mutation in a gene of interest by growing a population of mammalian cells comprising a gene of interest and a dominant-negative allele of PMS2. Nicolaides II is further cited as teaching identifying a cell that harbors a mutation in the gene of interest. (Paper 18, page 8, lines 4.) Bujard is cited as teaching the inducible expression of genes by adding or removing tetracycline.

The Office Action asserts that one of skill in the art would have been motivated to combine Nicolaides I or Nicolaides II with Bujard

> because it would have provided a [sic] regulated expression of the dominant negative PMS2 and such would have been desired because the continuous expression of the dominant negative PMS2 would have resulted in high rate of mutation in the genomic DNA of the cell resulting in transformation. It is noted that the dominant negative PMS2 expression was known to cause cancer at the time of the invention.

Paper 18, page 8, lines 15-20.

The Patent Office has the burden of establishing a *prima facie* case of obviousness. (MPEP § 2142.) To establish *prima facie* obviousness of a claimed invention there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. *In re Fine*, 837 F.2d 1071 (Fed. Cir. 1988); *In re Jones*, 958 F.2d 347 (Fed. Cir. 1992). The motivation to modify or

combine reference teachings must be found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. *In re Kotzab*, 217 F.3d 1635 (Fed. Cir. 2000).

None of Nicolaides I, Nicolaides II, nor Bujard provide any suggestion or motivation to one of ordinary skill in the art to modify a stable cell line expressing *PMS2-134* (as taught by Nicolaides I or Nicolaides II) such that the *PMS2-134* gene is under the control of a tetracycline inducible promoter (as taught by Bujard). Nicolaides I and Nicolaides II merely teach that *PMS2-134* is a dominant-negative mismatch repair gene and is useful for the production of eukaryotic mismatch repair cells and organisms with diverse phenotypes. Nicolaides I teaches that "the ability to inactivate endogenous MMR of cells through the introduction of the hPMS2-134 protein may have some practical value. In particular, it suggests a way to make other eucaryotic cells MMR deficient." (Page 1640, column 2, lines 13-16.) Nicolaides I also teaches that *hPMS2-134* "might facilitate the production of highly diverse agricultural and livestock products." (Page 1641, column 1, lines 7-8.) Nicolaides II similarly teaches, "By introducing these [dominant-negative mismatch repair] genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation." (Lines 3-6 of the Abstract) Neither Nicolaides I or II suggests the desirability of regulating expression of a *PMS2* dominant-negative mismatch repair gene in cells.

Bujard provides no suggestion or motivation to use a tetracycline-regulated gene expression system to regulate dominant-negative *PMS2* mismatch repair gene expression. Bujard only teaches that it is desirable to use the tetracycline-regulated gene expression

system to "turn off" expression of genes that are cytotoxic to cell lines. "[S]table cell lines carrying genes that are cytotoxic to the cells can be difficult or impossible to create due to 'leakiness' in the expression of the toxic genes. By repressing gene expression of such toxic genes using the transcriptional inhibitor fusion proteins of the invention, stable cell lines carrying toxic genes may be created." (Page 54, lines 12-16.) The dominant-negative *PMS-2* mismatch repair gene, however, is not cytotoxic. Thus Bujard provides no suggestion or motivation to modify the cell lines containing a dominant-negative *PMS2* gene taught by Nicolaides I and II.

Furthermore, the knowledge generally available to one of ordinary skill in the art would not have provided any motivation to modify Nicolaides I or Nicolaides II with Bujard. The Office Action asserts that the motivation would have been to prevent transformation, *i.e.* cancer, in cells that constitutively express dominant-negative *PMS2*. The amended claims, however, are drawn to ***in vitro*** methods for generating a mutation in a gene of interest. Cells growing *in vitro* do not cause cancer. Thus one of ordinary skill in the art would not have been motivated by a desire to prevent transformation or cancer in the cells of the claimed method. Thus no teaching in the cited references, Nicolaides I, Nicolaides II, and Bujard, or in the knowledge generally available to one of ordinary skill would have motivated one of ordinary skill in the art to combine the references to arrive at the claimed methods. Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 103(a).

7. Conclusion

Applicants submit that the claims are in proper form for allowance and are not rendered obvious by the cited art. Prompt allowance of the claims is respectfully requested.

Respectfully submitted,

Date: July 21, 2003

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EXHIBIT 1

Comparison of Mouse and Human PMS2 N-termini

Mouse ME**Q**T**E**G**V**STE**C**AKAI KPID**G**KSVHQICSGQ V**I**LSLSTAVKEL**I**EN S**V**DAGAT**T**IDL**R**LKD
Human ME**RA**E**SS**STE**P**AKAI KPID**R**KSVHQICSGQ V**V**LSLSTAVKEL**V**EN S**L**DAGAT**N**IDL**K**LKD

Mouse YGVDLIEVSDNGCGV EEENFEGL**A**LKHHTS KIQEFADLTQVETFG FRGEALSSLCALSDV
Human YGVDLIEVSDNGCGV EEENFEGL**T**LKHHTS KIQEFADLTQVETFG FRGEALSSLCALSDV

Mouse TISTCH**G**SA**S** VGT
Human TISTCH**A**SA**K** VGT

Differences shown in boldface

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Genetics

Elevated levels of mutation in multiple tissues of mice deficient in the DNA mismatch repair gene *Pms2*

(transgenic mice/supF/genetic instability/cancer)

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ABSTRACT **The *Pms2* gene has been implicated in hereditary colon cancer and is one of several mammalian homologs of the *Escherichia coli mutL* DNA mismatch repair gene. To determine the effect of *Pms2* inactivation on genomic integrity *in vivo*, hybrid transgenic mice were constructed that carry targeted disruptions at the *Pms2* loci along with a chromosomally integrated mutation reporter gene. In the absence of any mutagenic treatment, mice nullizygous for *Pms2* showed a 100-fold elevation in mutation frequency in all tissues examined compared with both wild-type and heterozygous litter mates. The mutation pattern in the nullizygotes was notable for frequent 1-bp deletions and insertions within mononucleotide repeat sequences, consistent with an essential role for PMS2 in the repair of replication slippage errors. Further, the results demonstrate that high rates of mutagenesis in multiple tissues are compatible with normal development and life and are not necessarily associated with accelerated aging. Also, the finding of genetic instability in all tissues tested contrasts with the limited tissue distribution of cancers in the animals, raising important questions regarding the role of mutagenesis in carcinogenesis.**

Hereditary nonpolyposis colorectal cancer (HNPCC) has been linked to germ-line mutations in the human homologs of *Escherichia coli* and yeast DNA mismatch repair genes (1–3). Recent work has revealed that mammalian cells contain multiple homologs of the *E. coli* MutS and MutL proteins, including MSH2, MSH3/DUG1/MRP1, and GTBP/MSH6 (1, 2, 4–6) and MLH1, PMS2, and PMS1 (1, 2, 7–9), respectively. Biochemical studies with mammalian or yeast cell extracts or partially purified proteins have shown that the MutS homologs can associate in heterodimers in at least two combinations (MSH2 with MSH3 and MSH2 with GTBP/MSH6), each with different activities and mismatch substrate specificities, suggesting both redundancy and divergence of function (1, 10, 11). Studies with the MutL homologs suggest that MLH1 and PMS2 form a functional heterodimer (12–14); other possible MutL complexes have yet to be characterized.

In addition, evidence is emerging that the mismatch repair gene homologs may participate in various other cellular functions, such as transcription-coupled repair (15), recombination (16), and even cell cycle regulation (17). Consequently, a deficiency in one of these homologs may disrupt genome stability in multiple ways.

Previous attempts to evaluate the specific *in vivo* role of each of the mammalian mismatch repair homologs have been based for the most part on comparisons of changes at microsatellite loci. However, these assays report only expansion or contraction of simple repeated sequences, representing just one specific subset of all possible genetic changes. Forward mutation assays, in contrast, are capable of detecting a wide range of mutations. Such assays have been carried out with the selectable *HPRT* locus in colon cancer-derived cell lines, some of which are deficient in DNA mismatch repair. These studies have revealed some significant differences among the mutation patterns in different lines (18, 19). However, such tumor cell lines are likely to contain multiple genetic abnormalities besides mismatch repair gene defects, and some of these might affect DNA metabolism, complicating interpretation of the experiments.

Hence, the full impact of *Pms2* inactivation on genomic integrity *in vivo* has not yet been elucidated. Herein we report the construction and analysis of hybrid transgenic mice carrying targeted disruptions at the *Pms2* loci along with a chromosomally integrated mutation reporter gene contained within a recoverable λ phage shuttle vector. Data are presented showing that mice nullizygous for *Pms2* have high levels of spontaneous mutagenesis in multiple tissues, even those not associated with an increased risk of cancer. In addition, *Pms2* inactivation is shown to predispose to deletions and insertions, with a significantly lesser impact on the occurrence of base substitutions.

MATERIALS AND METHODS

Transgenic Mice. Construction of the *Pms2*-deficient mice (using D3 embryonic stem cells of 129/Sv mouse origin) has been described (20). The 3340 *supFG1* mice were produced as described for the 1139 mice (21) except that the *supFG1* gene was substituted for *supF* in the λ vector. Both were derived from the C57BL/6 mouse background. Construction of the *supFG1* gene has been described (22). The *Pms2* genotype of the mice was determined by the presence or absence of the targeted insertion by PCR amplification of a region in the *Pms2* gene as described (20). The presence of the *supFG1* or *supF* gene in the mice was also confirmed by PCR amplification, as described (21) .

λ Shuttle Vector Rescue and Analysis. High molecular weight DNA was prepared from selected mouse tissues as described (21). λ vector rescue from the mouse DNA was carried out with λ *in vitro* packaging extracts (21, 23, 24). Packaging extracts were made as described (23), except that a new *E. coli* lysogen, NM759 [*E. coli* K12 *recA56* Δ(*mcrA*) e14° Δ(*mrr-hsd-mcr*) (λ*imm434 cIts b2 red3 Dam15 Sam7*)/λ] was used instead of BHB2690 for the preparation of the sonicate extract (24). This lysogen produces extracts that are deficient in methyl-directed restriction activity that would otherwise degrade DNA methylated in the mammalian

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Abbreviation: HNPCC, hereditary nonpolyposis colorectal cancer.

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pattern and reduce the yield of rescued phage (24, 25). The mouse DNA was incubated in the λ *in vitro* packaging extracts at a concentration of 0.05 μg/μl for 2 h at 37°C. The packaged phage were diluted in 10 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$, adsorbed to PG901 [*E. coli* C1a *lacZ125* (*am*)], and plated in 0.6% top agar on LB plates in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside (1.6 mg/ml) and isopropyl β-D-thiogalactoside (1.3 mg/ml), as described (23). Phage with functional *supF* genes suppress the nonsense mutation in the host bacteria β-galactosidase gene, allowing synthesis of active enzyme capable of metabolizing 5-bromo-4-chloro-3-indolyl β-D-galactoside, thereby yielding blue plaques. Phage with inactivating *supF* mutations produce colorless plaques.

Mutation Sequencing. After plaque purification, PCR amplification of the *supF* gene sequences was carried out with the GeneAmp kit (Perkin–Elmer/Cetus). Sequence analysis of the PCR products was performed as described (26).

RESULTS

Construction of Hybrid Transgenic Mice. To elucidate in a well-defined system the *in vivo* role of the MutL homologs, mice deficient for *Pms2* were constructed by gene targeting in embryonic stem cells (20). Initial studies of the *Pms2* nullizygotes revealed increased microsatellite mutation, sterility in males, and the development of lymphomas and sarcomas within the first year of age (20). To study genetic instability in multiple tissues from these mice, we designed an *in vivo* forward mutation assay. The *Pms2*-deficient mice were bred with transgenic mice carrying the *supF* tRNA suppressor gene as a mutation reporter gene within a chromosomally integrated recoverable λ phage shuttle vector (21, 23). By using λ *in vitro* packaging extracts, the vector DNA can be identified, excised, and packaged from within the mouse DNA into viable λ particles for analysis in bacteria of *supF* mutations that occurred in the animals (23, 24). These *supF* mice therefore provide an *in vivo* mutation reporter system that can detect base substitutions, as well as deletions and insertions. Consequently, this system for reporting mutations is more sensitive and complete than the standard PCR-based assay of microsatellite stability.

To enhance the sensitivity of the assay even further, we used two independent lines of *supF* mice, one (1139) carrying the standard *supF* gene (21) and another (3340) with a *supF* gene modified to contain extended simple sequence repeats (*supFG1*) (22). Male *supF*-positive mice were bred with *Pms2* heterozygous females to construct hybrid transgenic mice. The F_1 mice were crossed to produce F_2 mice that were wild type, heterozygous, or nullizygous at the *Pms2* loci and that also carried the *supF* transgene.

Analysis of Spontaneous Mutagenesis *in Vivo*. Three independent sets of 3340/*Pms2* male litter mates were identified for analysis at 12 weeks of age. The mice were maintained under standard animal husbandry conditions in the same cage. There was no known exposure to any mutagenic or genotoxic agent. DNA was prepared from several different tissues of the mice and used for vector rescue (via *in vitro* packaging) and reporter gene analysis (Table 1).

The frequency of mutations in the wild-type mice was in the range of 1 to 2 × 10^{-5}, consistent with previous observations of baseline mutation frequencies in such transgenic animal systems (21, 25, 27). However, the nullizygote samples consistently showed 100-fold or more elevations in mutation frequencies in all tissues and mice tested (Table 1). Significant tissue to tissue differences were not detected, with the largest difference being 2-fold (between liver and colon in F2–19). However, colon in F2–29 showed a slightly lower frequency than in F2–19, and there were similar slight fluctuations in the data from the skin samples from the three nullizygous mice. Analysis of the heterozygotes revealed mutation frequencies similar to those found in the wild-type animals.

Table 1. Spontaneous mutagenesis of the *supFG1* transgene in *Pms2*-deficient 3340 mice

Mouse/tissue	Mutation frequency (×10^{-5})	No. mutants/ total no.
Wild type		
Mouse F2-17		
Skin	≤2	0/72,990
Liver	4	6/170,260
Mouse F3-9		
Skin	≤2	0/72,240
Colon	2	1/45,620
Total	2	7/361,110
Heterozygous		
Mouse F2-18		
Skin	5	5/92,730
Liver	4	5/131,500
Spleen	≤2	0/75,630
Colon	≤2	0/78,620
Mouse F2-4		
Skin	1	1/87,690
Mouse F3-13		
Skin	2	1/51,600
Total	2	12/517,760
Nullisygous		
Mouse F2-19		
Skin	237	302/127,700
Liver	132	55/41,700
Spleen	226	33/14,620
Colon	286	201/70,360
Brain	270	53/19,610
Lung	206	35/16,980
Mouse F2-29		
Skin	177	27/15,270
Spleen	182	27/14,850
Colon	187	37/19,780
Mouse F3-11		
Skin	173	58/33,590
Liver	118	54/45,650
Total	210	882/420,110

In the 1139/*Pms2* hybrids, the nullizygotes showed only a 5-fold overall increase in mutation frequency (Table 2). However, the 1139 mice were previously found to have an unusually high frequency of spontaneous deletions due to locus-specific effects at the transgene integration site (21). When only *supF* point mutations are considered, the nullizygotes again show a substantial elevation in mutation frequency of approximately 25-fold relative to the wild-type mice (Table 2).

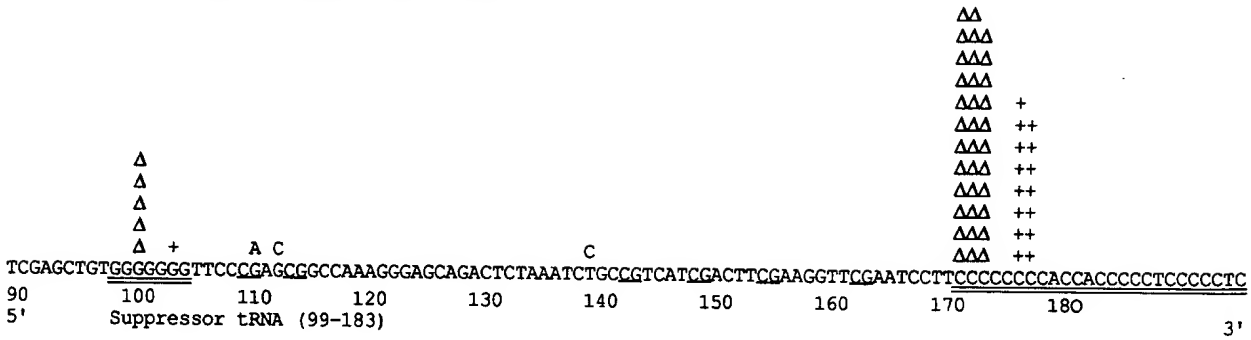
Mutation Patterns. The mutations arising in the nullizygous mice were analyzed at the sequence level (Fig. 1 and Table 3). Almost all of the mutations in the 3340/*Pms2* nullizygotes (71 out of 74) were single-base pair deletions or insertions within the G·C base pair repeats at positions 99–105 and 172–179. The eight G·C base pair run at positions 172–179 is a particular hot spot. Note that this stretch constitutes a subset of a longer G·C-rich region that extends beyond the end of the tRNA gene at position 183. This pattern was seen in multiple tissues in three different nullizygous mice from three different litters (Fig. 1*A*).

In the 1139 mice, single-base pair insertions and deletions were less prominent (6 of 18 mutations) but were still more frequent than in the wild-type mice (0 of 30 mutations) (Fig. 1*B* and Table 3). The difference in mutation pattern between the 3340 and the 1139 mice can be attributed in part to the differences in the reporter genes. *SupFG1* in 3340 mice has two runs of 7 and 8 G·C base pairs, respectively. The longest

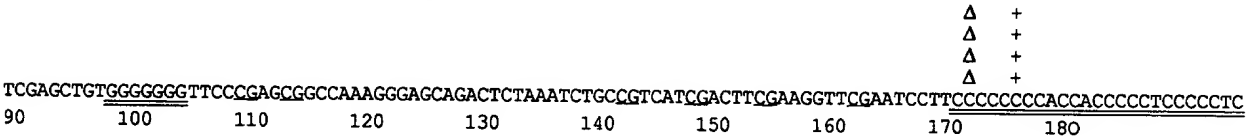
A.

3340/*PMS2* nullizygotes

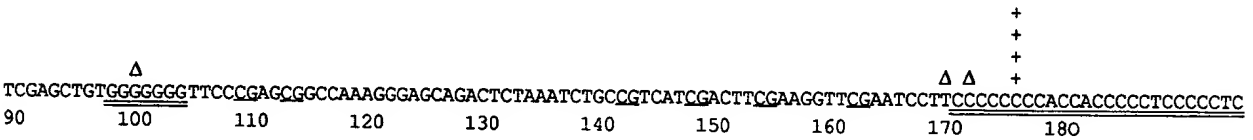
Mouse F2-19 (skin, liver, and colon)



Mouse F2-29 (skin, liver, colon)

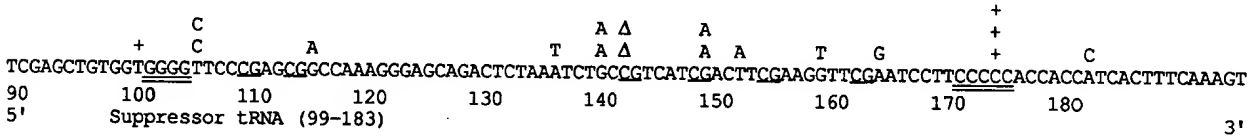


Mouse F3-11 (skin)



B.

1139/*PMS2* nullizygotes



1139 wild type

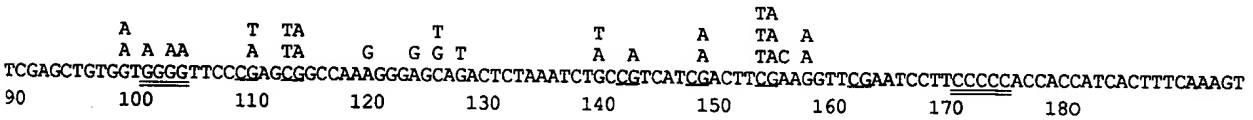


FIG. 1. Sequences of reporter gene mutations in *Pms2* nullizygous mice. (*A*) Mutations in the *supFG1* gene in 3340/*Pms2* nullizygotes. Mutations from three different mice from independent litters are shown, with the tissues of origin indicated. Base substitutions are listed above the original sequence, and single-base pair deletions or insertions are indicated by Δ or +, respectively, above the corresponding site. Mononucleotide repeat sequences are highlighted by double underlining. CpG sequences, potential sites of cytosine methylation, are noted by a single underline. (*B*) Mutations in the *supF* gene in 1139/*Pms2* nullizygotes and in wild-type 1139 mice, as indicated.

repeat sequence in the *supF* gene in the 1139 mice, however, is a 5 G·C base pair stretch at positions 171–176. We propose that this shorter region is less susceptible to slippage replication errors (28) and is, therefore, less dependent on *Pms2* function for stability.

The base substitutions in the nullizygotes were mostly transitions (Table 3). Because there were so few base substitutions in the 3340/*Pms2* nullizygotes (only 3 of 74 mutations), the main comparison to be made is between the 1139/*Pms2* nullizygotes and the wild-type mice. Overall, the spectra of base substitutions are similar except that the proportion of C → T transitions at CpG sites in the nullizygotes is only 11%, compared with 47% in the wild type. Because CpG sites are subject to cytosine methylation in mammalian cells, transitions at these positions are thought to arise from the propensity of 5-methylcytosine to deaminate

Table 2. Spontaneous mutagenesis of the *supF* transgene in *Pms2*-deficient 1139 mice

Mouse/tissue	Mutation frequency ($\times 10^{-5}$)	No. mutants/total no.	% point mutations	% deletions	Frequency of point mutations ($\times 10^{-5}$)
Wild type					
Skin	39	199/511,950			
Liver	29	47/199,010			
Spleen	35	82/232,580			
Total	35	328/943,540	6	94	2
Nullizygous					
Skin	117	163/138,910			
Liver	139	165/118,860			
Spleen	154	151/98,360			
Total	135	479/356,120	42	58	57

to thymidine. Although such mutations do occur in the *Pms2* nullizygotes, they are under-represented relative to those in the wild-type animals, suggesting that *Pms2* is not essential for repair of these lesions. This is consistent with reports of an alternate repair pathway involving a glycosylase specific for the G·U and G·T mismatches that are produced by deamination (29, 30). The paucity of methylcytosine-related transitions in the nullizygotes is not due to undermethylation of the transgene sequences. The *supF* transgenes in both the 3340 and 1139 mice are each heavily methylated, as determined by comparison of susceptibility of the mouse genomic DNA to *Hpa*II and *Msp*I digestion (data not shown).

DISCUSSION

We have detected high somatic mutation frequencies in *Pms2* nullizygous mice of up to 100-fold above the wild-type background. Mice heterozygous for the *Pms2* disruption did not show increased spontaneous mutagenesis, suggesting that a single functional allele provides near normal repair activity and that *Pms2*-related genetic instability may, therefore, be recessive. However, we cannot rule out the possibility that the heterozygotes do have a low level of genetic instability, since slight increases in mutation frequency (such as 2-fold or less) are difficult to reliably detect in this assay, even with the analysis of large numbers of animals and phage. In light of the apparent participation of mismatch repair factors in aspects of DNA repair other than mismatch correction (15, 31), differences between the heterozygotes and the wild-type mice may eventually be revealed by studies of induced mutagenesis.

The heterozygous mice mimic the situation in most patients with the HNPCC syndrome, who are also heterozygous at one of the mismatch repair loci in their somatic cells (1, 3). Their tumors, however, are characterized by loss or inactivation of the second allele, leading to genomic instability. Such loss of heterozygosity events should also be possible in mice, thus predisposing the affected cell lineages to *supF* mutations. However, the low mutation frequencies in the heterozygotes suggest that such events are rare.

Table 3. Mutations in *Pms2* nullizygous mice

Mutation	3340/PMS2 nullizygotes, no.	1139/PMS2 nullizygotes, no.	1139 wild-type mice, no.
C·G → T·A	1	5	23
T·A → C·G	1	3	2
C·G → A·T	0	1	3
C·G → G·C	1	0	1
T·A → A·T	0	2	0
T·A → G·C	0	1	1
C·G → T·A at CpG	1	2	14
+1 insertion	24	4	0
−1 deletion	47	2	0
Total	74	18	30

The spectrum of mutations in the nullizygotes is consistent with a primary role for *Pms2* in the correction of slippage errors occurring during replication of repeated sequences (28). By comparison of the results in the *supF* and *supFG1* reporter genes, we can conclude that seven or more mononucleotides are particularly unstable *in vivo*. This is consistent with the frequent finding of frameshift mutations within a 10 A·T base-pair run in the transforming growth factor β receptor gene in a series of colon cancer cell lines (32, 33). Such long mononucleotide runs are also particularly common within promoters and introns. For example, the *c-myc* promoter contains several extended runs of G·C base pairs (34), and the binding site for the Sp1 transcription factor is highly G+C-rich (35). We would predict that these regulatory regions would be especially unstable in the setting of *Pms2* deficiency, leading to accumulating abnormalities in gene expression and thereby contributing to aberrant cellular function and eventually carcinogenesis.

The reduced frequency of C → T transitions at CpG sites in the *Pms2* nullizygotes is in contrast to the results of Andrew and colleagues (S. E. Andrew, A. H. Reitmar, J. Fox, L. Hsiao, A. Francis, M. McKinnon, T. W. Mak, and F. Jirik, personal communication), who have found a higher frequency of such mutations in mice deficient in the MutS homolog *Msh2*. Since they used a different mutation reporter gene (*lacI*), the discrepancy could be attributed to the known propensity for such mutations in the *lacI* transgene (27). Also, the different loci of integration of the reporter genes may influence the mutation pattern, as has occasionally been seen in transgenic constructs (21, 36). It is also possible, however, that *Msh2* has a role in the repair of the G·T mismatches that result from deamination of methylcytosine.

In addition, the mutation frequency in the *Msh2*-deficient mice studied by Andrew *et al.* (personal communication) is somewhat less than that seen in the *Pms2* nullizygotes. Some of this difference may again be explained by differences in the reporter genes. Nonetheless, the comparsion indicates that *Pms2* deficiency results in a degree of genetic instability at least as severe as that caused by lack of *Msh2*. Hence, the observation that *Msh2* mutations are more common in HNPPC patients than are *Pms2* mutations (2, 8) cannot be attributed to a greater impact of *Msh2* mutations on genetic instability. Possibly, it could relate to differences in the mutation spectra, although these differences need to be confirmed in other target genes. More likely, *Msh2* mutations may be more prevalent because of other factors, such as an increased susceptibility of the *Msh2* locus itself to mutation or to loss of heterozygosity.

The nullizygous mice showed increased mutation in all tissues tested, including skin, liver, spleen, colon, brain, and lung. However, these animals appear to be predisposed to

only lymphomas and sarcomas (20). This disparity shows that mutagenesis is just one element in carcinogenesis, highlighting the complexity of cancer etiology. Additional factors could include tissue damage and aberrant mitogenic stimuli. However, the particular factors that influence the tissue distribution of neoplasms in the mice remain to be determined. In HNPCC patients, the range of tumors is also limited; but it is different from that seen in the mice, with epithelial cancers of the colon most common. One explanation for this difference could be that most of the HNPCC patients are genotypically heterozygotes, and inactivation of the second mismatch repair gene allele is a critical step in the progression to neoplasm (1, 3, 37). Tissue-specific factors that vary among species, such as differential exposure to diet-derived genotoxic agents in the colon, may play an important part in promoting the loss of heterozygosity.

Several unusual patients, however, have been found to lack mismatch repair activity in their nonneoplastic tissues and in their cancers (38, 39). Two of these were determined to be heterozygous at the *Pms2* locus, but in each case the mutant allele was found to code for a truncated protein with apparent dominant negative activity. In terms of *Pms2*-related mismatch repair function, the phenotype of these patients is, therefore, similar to that of the *Pms2*-nullizygous mice; yet, they developed colorectal carcinomas, not lymphomas or sarcomas. This difference suggests that there may be additional species-specific factors influencing tumor distribution. Determining whether these factors are genetic in nature (such as differences in the controls on growth and differentiation) or reflect environmental and life style differences will require further study.

Nonetheless, the *Pms2* nullizygous mice described here show, remarkably, that high levels of spontaneous somatic mutations are compatible with mostly normal development and life, except for early onset carcinogenesis and male infertility (20). It might be predicted that such profound genetic instability would also cause accelerated aging, but this has not yet been demonstrated in the mice. Recently, Werner syndrome, a disease of premature aging and predisposition to malignancy, has been linked to a gene encoding a putative DNA helicase (40). In this disorder, the lack of helicase activity is hypothesized to lead to the accumulation of genetic abnormalities, producing early cellular senescence. However, mutations in Werner cells include a high proportion of large deletions (41), rather than point mutations as seen in the *Pms2* mice. Whether this difference in mutation pattern accounts for the differences in carcinogenesis and aging remains to be determined.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Applicati n of:

Nicolaides *et al.* **Confirmation No.:** 6480

Serial No.: 09/853,646 **Group Art Unit:** 1632

Filing Date: May 14, 2001 **Examiner:** R.R. Shukla

For: A METHOD FOR GENERATING HYPERMUTABLE ORGANISMS

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration under 37 CFR 1.131

I, Nicholas C. Nicolaides, hereby state the following:

1. I earned my doctorate in Genetics at Thomas Jefferson University in Philadelphia, Pennsylvania.

2. I am a founder of Morphotek Inc., and serve as President, Chief Executive Officer and Chief Science Officer.

3. I am an inventor of the above-referenced patent application and am thoroughly familiar with the field of molecular biology, and in particular, mismatch repair.

4. In accordance with the teachings of our specification, we have generated a dominant negative mutant form of a plant PMS2 from *Arabidopsis thaliana*.

5. The following describes the experiments for expression of an *Arabidopsis thaliana PMS2* truncation mutant in bacteria:

An *Arabidopsis thaliana* dominant negative MMR gene mutant was created by generating a construct with similar domains to that of the human dominant negative PMS2 gene (referred to as hPMS2-134). To generate this vector, the *A. thaliana* PMS2 (ATPMS2) and human PMS2 (hPMS2) cDNA sequences were aligned and the conserved domain was isolated. Figure 1 shows a sequence alignment between the human and *A. thaliana* PMS2-134 amino acid sequences wherein a 51% identity is found between the

two sequences. Dominant negative hPMS2-134 and ATPMS2-134 genes were made by PCR and subcloned into bacterial expression vectors.

Recombinant clones were sequenced to ensure authentic sequences. Inserts were then cloned into the inducible pTAC expression vector, which also contains the ampicillin resistance gene as a selectable marker. The hPMS2-134 allele was also cloned into the pTAC expression vector as a positive control. Electrocompetent DH5alpha and DH10B bacterial cells (Life Technologies) were electroporated with empty vector, and the loaded vectors pTACATPMS2-134 and pTAChPMS2-134, using an electroporator (BioRAd) following the manufacturer's protocol. Bacterial cultures were then plated on to LB agar plates containing 100 pg/ml ampicillin and grown at 37°C for 14 hours. Ten recombinant clones were then isolated and grown in 5 mls of LB broth containing 50 pg/ml ampicillin plus 50 pM IPTG for 18 hr at 37°C. One hundred microliters were then collected, spun down, and directly lysed in 2X SDS buffer for western blot analysis. For western analysis, equal number of cells were lysed directly in 2X SDS buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes.

Lysate proteins are separated by electrophoresis on 4-12% NuPAGE gels (Novex). Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a polyclonal antibody generated against MMR polypeptide sequence or a fused tag (*e.g.*, FLAG, HIS, *etc.*) and a horseradish peroxidase conjugated secondary antibody, using chemiluminescence for detection (Pierce). Both hPMS2-134 protein and ATPMS2-134 protein were expressed, whereas cells expressing empty vector had no detectable expression (data not shown).

Bacterial clones expressing the hPMS2-134, ATPMS2-134 or the empty vector were grown in liquid culture for 24 hr at 37°C in the presence of 50 pg/ml ampicillin plus 50 pM IPTG. The next day, cultures were diluted 1:10 in medium containing 50 pM IPTG plus ampicillin or ampicillin plus 25 pg/ml kanamycin (AK) and cultures were grown for 18 hr at 37°C. The following day, a 0.1 ul aliquot (2 ul diluted in 1000 ul of LB medium and used 50 ul for plating) of cells grown in Amp medium were plated on LB agar plates containing 40 ug/ml of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) plus 100 pg/ml ampicillin (AMP), while a 1 ul aliquot (1 ul diluted in 100 1 of LB medium and used 100 ul for plating) of cells grown in AK medium were plated on LB-agar plates containing X-gal and 50 pg/ml kanamycin (KAN). Plates were incubated for 18 hours at 37°C.

The results from these studies show that cells expressing the hPMS2-134 or the ATPMS2-134 polypeptides displayed increased mutation rates in the genome of the DH10B bacterial strain which resulted in the production of KAN resistant clones (Figure 2). Bacterial cells expressing the ATPMS2-134 were found to have an increase in the number of KAN resistant cells (12 clones) in contrast to cells expressing the empty vector, which yielded no KAN resistant clone. These data demonstrate that dominant

negative ATPMS134 MMR genes are useful for creating hypermutable organisms that can generate phenotypically diverse offspring.

6. All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

7. I hereby acknowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001, and may jeopardize the validity of the application or any patent issuing thereon.

Nicholas C. Nicolaides

6-17-03
Date

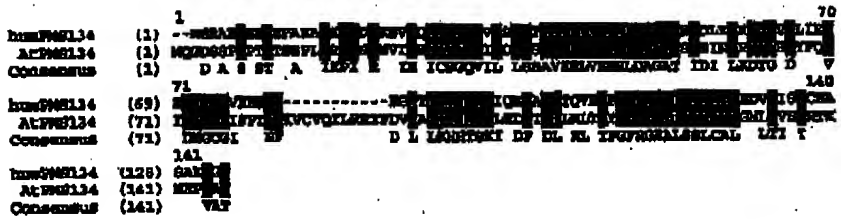

Figure 1: Alignment between human PMS2-134 (hPMS2-134) and *Arabidopsis thaliana* homolog of PMS2-134 (ATPMS2-134) amino acid sequences. Similarity is 65.1%, identity is 50.7%. Black boxes show identical residues.

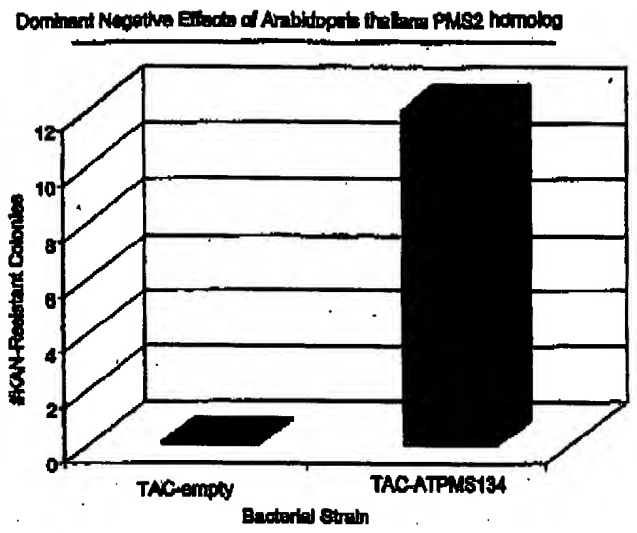

Figure 2: Expression of the *Arabidopsis thaliana PMS2-134* gene produces hypermutability in bacteria leading to the generation of new phenotypes. Bacterial cultures expressing the *ATPMS2-134* gene resulted in a genetic alteration of the bacterial host and the generation of clones that are kanamycin resistant.

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Functional Domains of the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA Mismatch Repair Proteins and Their Relevance to Human Hereditary Nonpolyposis Colorectal Cancer-Associated Mutations

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The MutL protein is an essential component of the *Escherichia coli* methyl-directed mismatch repair system but has no known enzymatic function. In the yeast *Saccharomyces cerevisiae*, the MutL equivalent, an Mlh1p and Pms1p heterodimer, interacts with Msh2p bound to mismatch-containing DNA. Little is known of the functional domains of Mlh1p and Pms1p. In this report, we define the Mlh1p and Pms1p domains required for Mlh1p-Pms1p interaction. The Mlh1p-interactive domain of Pms1p is comprised of 260 amino acids near the carboxyl terminus while the Pms1p-interactive domain of Mlh1p resides in the final 212 residues. The two domains are sufficient for Mlh1p-Pms1p interaction, as determined by the two-hybrid assay and by in vitro protein affinity chromatography. Deletions within the domains completely eliminated Mlh1p-Pms1p interaction. Using site-directed mutagenesis, we altered a number of highly conserved residues in the Mlh1p and Pms1p proteins, including some alterations that mimic germline mutations observed for human hereditary nonpolyposis colorectal cancer. Alterations either in the consensus MutL box located in the amino-terminal portion of each protein or in the carboxyl-terminal homology motif of Mlh1p eliminated DNA mismatch repair function but had no effect on Mlh1p-Pms1p interaction. In addition, certain *MLH1* and *PMS1* mutant alleles caused a dominant negative mutator effect when overexpressed. We discuss the implications of these findings for the structural organization of the Mlh1p and Pms1p proteins and the importance of Mlh1p-Pms1p interaction.

In both prokaryotes and eukaryotes, DNA mismatch repair (DMR) plays several roles in DNA metabolism, including the repair of mispaired bases that result from DNA replication, repair of DNA mismatches that arise due to spontaneous deamination of 5-methylcytosine or chemical base damage, and repair of mispairs that form during genetic recombination (40). In humans, mutations in the DMR genes *MSH2*, *MLH1*, *PMS2*, and *PMS1* are associated with hereditary nonpolyposis colorectal cancer (HNPCC) (12, 18, 29, 43, 44, 55; reviewed in reference 26). Mutation of DMR genes has also been observed at a variable frequency in a number of sporadic tumors (26, 33, 52). In mice, disruptions of the mouse DMR genes *MSH2*, *PMS2*, and *MLH1* result in microsatellite instability and increased tumor development (6, 7, 14, 49). Additionally, *PMS2*- and *MLH1*-deficient mice display abnormalities in meiotic chromosome synapsis and reciprocal exchange, respectively (6, 7, 16).

Biochemical studies with *Escherichia coli* indicate that methyl-directed DMR is initiated through the action of three proteins, MutS, MutL, and MutH (39). The MutS protein recognizes DNA mismatches, and MutH is an endonuclease that directs repair to the newly synthesized strand (39). Although no biochemical activity of MutL has been demonstrated, the MutL protein appears to couple mismatch recognition by MutS to MutH activation in an ATP-dependent manner. Furthermore, DNase I protection experiments have shown that the MutL protein interacts with a MutS-heteroduplex DNA complex in the presence of ATP (20). In the yeast *Saccharomyces cerevisiae*, DMR employs three MutS homologs, Msh2p, Msh3p, and Msh6p (10, 26, 36, 41), and two MutL homologs, Mlh1p and Pms1p (27, 28, 46). In both yeast and human, Msh2p appears to form heterodimers with either Msh3p or Msh6p, with the two heterodimer species displaying different DNA mismatch recognition specificities (1, 2, 15, 22, 23, 25, 36, 43). We have previously reported that Mlh1p and Pms1p form a heterodimer or higher-order multimer that binds to an Msh2p-heteroduplex DNA complex in vitro (47). In vivo, additional factors are likely to interact with Mlh1p and Pms1p during the DMR process. Recent studies have identified one such factor, the DNA replication processivity factor PCNA. Those studies suggested that PCNA interacts with human PMS2 (hPMS2) and, in the case of yeast, with both Mlh1p and Msh2p and that PCNA is required for an early step in mismatch repair (54). This interaction suggests a tight linkage between the DNA replication apparatus and DMR, possibly reflecting one aspect of the strand discrimination mechanism.

Despite our growing understanding of the role of DMR mutations in human and mouse tumorigenesis, little is known regarding the functional domains of DMR proteins. Previous work has shown that the bacterial MutS protein contains an ATP binding domain and that mutations in this domain alter both the ATPase and the mismatch binding activities of MutS (21, 57). Recent studies with yeast have shown that site-directed mutagenesis of the ATP binding domain of Msh2p did not affect initial mismatch recognition but caused a dominant negative effect when overexpressed in wild-type cells (4). A screen for dominant negative alleles of the bacterial mutL gene has identified several amino acid substitutions, mainly in the amino-terminal portion of the protein (5). In humans, specific

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mutations in two hMutL homologs, hMLH1 and hPMS2, apparently result in altered proteins that abolish DMR, possibly in a dominant negative manner, and lead to early tumor development (45). Here we report the use of site-directed mutagenesis and two-hybrid assays to determine functional domains in yeast Mlh1p and Pms1p that are critical for DMR, heterodimerization, and possibly interaction with other proteins. Additionally, our results provide a framework for understanding the consequences of specific DMR mutations observed in human cancer and provide rationales for identifying additional components in the process.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* DH1 was used for all plasmid constructions. Maltose binding protein (MBP) fusion proteins were expressed in *E. coli* PR78 (*lon*::Tn*10Δ16Δ17 argE*(Am) Spc^r Rif^s *araD139 galE galK phoAΔ20 thi*). The yeast two-hybrid reporter strain L40 of *S. cerevisiae* (56) was used for the in vivo interaction studies. For mutational analysis and overexpression of hemagglutinin 1 (HA1)-tagged proteins, the yeast strain MW3317-21A and its *mlh1Δ* and *pms1Δ* derivatives were used (46). Yeast cells were grown on YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, 2% agar) or appropriate selective "dropout" medium (0.7% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 2% agar, and 0.09% dropout mix lacking the amino acid used for selection).

Plasmid constructions and site-directed mutagenesis. (i) Plasmid constructs. All DNA manipulations were carried out by using standard methods (34). Plasmids pGAD-yMLH1, pGAD-yPMS1, pBTM-yMLH1, and pBTM-yPMS1 were constructed by cloning the coding sequences for the yeast Mlh1p and Pms1p proteins into the two-hybrid vectors pGAD424 (8) and pBTM116 (56), respectively.

For constructing the yeast *MLH1* complementation plasmid pCOM-yMLH1, an *Sph*I-*Sma*I fragment in yeast vector YCp50 containing the *URA3* gene was replaced with a *TRP1*-containing *Sph*I-*Pvu*II fragment from pBTM116. This created plasmid YCp-TRP1. Then an *Sph*I-*Pvu*II fragment containing the yeast *MLH1* gene from the original YEp24-yMLH1 plasmid (46) was inserted into the *Sph*I and *Eco*RI (blunted) sites of YCp-TRP1. The resulting plasmid only partially complemented the *mlh1Δ* strain. Therefore, an *Xba*I (blunted)-*Cla*I fragment of the 5′ *MLH1* sequence (46) was inserted into the partial-complementation plasmid, predigested with *Nae*I in YCp-TRP1 and *Cla*I in the *MLH1* gene, to create plasmid pCOM-yMLH1, which fully complements the *mlh1Δ* strain.

Plasmids pMAL-yMLH1 and pMAL-yPMS1 have been previously described (47). The pMAL constructs expressing residues 1 to 265 of Mlh1p [Mlh1p(1–265)], Mlh1p(501–769), Pms1p(1–271), and Pms1p(692–904) were prepared by subcloning the corresponding cDNA fragments (see "Deletion mutants" below) into the multiple-cloning site of the pMAL vector.

MLH1 and *PMS1* constructs, used to test for dominant negative effect and protein stability, were created in pCoB-5006 and pJAS. pCoB-5006 was constructed as follows. A 405-bp *Sph*I (blunted)-*Hin*dIII fragment containing the alcohol dehydrogenase I (ADH) gene promoter and a 230-bp *Eco*RI-*Sph*I (blunted) fragment containing the ADH gene terminator from pBTM116 were inserted into pBluescript II SK$^-$ (Stratagene) at the *Hin*cII-*Hin*dIII and *Eco*RI-*Sph*I (blunted) sites, respectively, to create pADH. This ADH promoter/terminator cassette was used to replace the fragment containing the ADH promoter-*lexA*-ADH terminator sequence in pBTM116 by digesting pADH with *Bss*HII and pBTM116 with *Sph*I and end filling both the pBTM116 vector and the ADH promoter/terminator cassette with T4 DNA polymerase. pJAS was created by PCR amplifying the ADH promoter sequence from yeast genomic DNA and replacing the 420-bp *Xho*I-*Eco*RI ADH promoter fragment in pCoB-5006 with the PCR fragment. This step created an *Nco*I site and an ATG start codon at the 3′ end of the ADH promoter. *Bam*HI-*Sal*I fragments encoding full-length (wild type or with point mutations) and amino-terminal portions (see "Deletion mutants" below) of Mlh1p or Pms1p were cloned into the corresponding sites of pCoB-5006. For carboxyl-terminal Mlh1p and Pms1p constructs, the truncated Mlh1p- and Pms1p-encoding fragments were fused in frame with the ATG start codon in pJAS.

Epitope-tagged constructs were made by insertion of triple tandem HA1 epitope sequences as described previously (50). Briefly, PCR primers were used to introduce a *Not*I site immediately upstream of the termination codon of full-length or truncated *MLH1* or *PMS1* (in the pCoB vector) and a *Sal*I site at the end of the HA1 triple peptides (53). Then, a *Not*I-*Sal*I fragment containing the HA1 triple tag was cloned into the corresponding site of the modified *MLH1* or *PMS1* plasmid.

(ii) Deletion mutants. Deletion derivatives of *MLH1* were generated by using the following naturally occurring restriction sites: *Bgl*II at position 2279–2284 [Mlh1p(1–761)], *Xmn*I at position 2193–2202 [Mlh1p(1–731)], *Sca*I at position 1885–1890 [Mlh1p(1–629)], *Bsu*36I at position 1506–1512 [Mlh1p(1–502) and Mlh1p(501–769)], *Spe*I at positions 794–799 [Mlh1p(1–265)] and 1193–1198 [Mlh1p(397–769)], *Nde*I at position 376–381 [Mlh1p(1–126)], and *Hin*dIII at position 1665–1670 [Mlh1p(555–769)]. For the Pms1p deletions, the internal restriction sites used to generate the truncated proteins were as follows: *Nco*I at position 2649–2654 [Pms1p(1–881)], *Hpa*I at position 2091–2096 [Pms1p(1–693) and Pms1p(692–904)], *Spe*I at position 1588–1593 [Pms1p(1–525) and Pms1p(524–904)], *Bsm*I at position 827–833 [Pms1p(1–271)], and *Sph*I at position 2208–2213 [Pms1p(735–904)]. A stop codon was generated at the 3′ deletion endpoint of each Mlh1p and Pms1p derivative by fusion to a linker, AGGCCT TAATTAATTAATTAATTAATTAAGTCGAC. For making Pms1p(1–152), the K153 codon AAG was changed to TAG by PCR-mediated mutagenesis (37).

(iii) Point mutations. All amino acid substitutions in the Mlh1p protein were introduced by PCR mutagenesis (37) with oligonucleotides encoding the following mutations. For changing alanine (A) to phenylalanine (F), glycine (G), or serine (S) at position 41, the forward primer, encoding amino acid residues 36 to 45 of MLH1, was used to modify the protein at position 41 to phenylalanine, glycine, or serine. The 268-bp PCR-generated fragments encoding the A-to-F, A-to-G, and A-to-S substitutions were used to replace the corresponding fragments in pCOM-YMLH1 and pGAD-YMLH1 to create the mutants A41F, A41G, and A41S, respectively.

To mutagenize the MutL box GFRGEAL of the Mlh1p protein, the *Bam*HI-*Acc*I fragment from pGAD-YMLH1 was subcloned into pBluescript SK$^-$ (Stratagene) to create pYMLH1-BA. The sequence corresponding to residues 37 to 100 of Mlh1p with *Cla*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively, was amplified by PCR. The reverse primer encoding amino acid residues 94 to 102 of Mlh1p, having a new codon in place of the codon for phenylalanine, arginine (R), or glycine, was used to modify the protein at position 96, 97, or 98, respectively, to alanine or valine (V). The 187-bp PCR products digested with *Cla*I and *Hin*dIII were ligated into *Cla*I- and *Hin*dIII-digested pYMLH1-BA. Then the *Bam*HI-*Nde*I or *Cla*I-*Nde*I fragment from the resulting plasmids was used to replace the corresponding fragment in pGAD-YMLH1 or pCOM-YMLH1 to create the mutants F96A, R97A, G98A, and G98V. To create the Pms1p MutL box mutant Pms1p-F126A, recombinant PCR (34) was used to modify the *PMS1* gene in pGAD-YPMS1. Primers in both the 5′-to-3′ and the 3′-to-5′ directions, encoding amino acid residues 122 to 130, were used to change the phenylalanine residue at position 126 to alanine. The same technique was employed to create Mlh1p-K619del, produced by an in-frame deletion of codon K619. In this case, primers in both the 5′-to-3′ and the 3′-to-5′ directions, encoding amino acid residues 614 to 624, were used to delete codon K619.

The point mutation constructs encoding the carboxyl-terminal homology (CTH) motif (final 13 amino acids) of Mlh1p were created in pMAL-YMLH1. Briefly, a 524-bp sequence, which encompassed codons for the final 10 amino acids of Mlh1p and the conjunctive 3′ untranslated region, was PCR amplified by using a forward primer that included a single point mutation and a *Bgl*II site and by using a reverse primer with another *Bgl*II site. This PCR product was inserted into the *Bgl*II site in pMAL-YMLH1. *Bsu*36I-*Sal*I and *Cla*I-*Sal*I fragments from pMAL-YMLH1, each encoding a K764-to-E or -R, F766-to-A, E767-to-D, or C769-to-A or -S substitution, were used to replace the corresponding fragments in pGAD-YMLH1 and pCOM-YMLH1, respectively, to create the mutants K764E, K764R, F766A, E767D, C769A, and C769S. All mutation-containing alleles were subjected to sequence analysis to confirm that the desired changes had occurred.

Yeast transformation and β-galactosidase assays. Yeast transformation was performed by the polyethylene glycol-lithium acetate method (19). For the color filter assay, colonies with pairing hybrids were lifted on nitrocellulose filters (Schleicher and Schuell BA-S85), frozen in liquid nitrogen, and incubated at 30°C on filter paper soaked with Z buffer (38). To measure β-galactosidase activity, cells were resuspended in 0.5 ml of Z buffer with 0.64 mg of *o*-nitrophenyl-β-D-galactosidase substrate per ml and permeabilized with chloroform. Reactions were stopped with Na_2CO_3 when an appropriate level of color had developed. β-Galactosidase activity was calculated by the equation: U/h = $[(OD_{420}/OD_{600}) \cdot 60]$/min, where OD_{420} and OD_{600} are the optical densities at 420 and 600 nm.

Measurement of mutation rates. To measure the reversion rates of the *hom3-10* allele, wild-type MW3317-21A and its *mlh1Δ* and *pms1Δ* derivatives with various complementation plasmid constructs were grown to saturation in minimal medium lacking tryptophan. Cells were then plated on selective medium lacking both tryptophan and threonine. Colonies appearing after 3 days of growth at 30°C were counted. The rate of *hom3-10* reversion was determined by fluctuation analysis as previously described (3, 32, 46). For each mutation rate, 12 independent cultures were tested.

In vitro protein interactions. The expression and purification of the MBP fusion proteins were performed as recommended by the manufacturer (New England BioLabs). Protein concentrations were determined by Bradford analysis (11) with bovine serum albumin as a standard. [^{35}S]methionine-labeled proteins were prepared by in vitro transcription and translation, using the rabbit reticulocyte lysate system (Novagen). Twenty microliters of [^{35}S]methionine-labeled proteins were added to MBP fusion protein columns. After washing with 10 volumes of buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA), the bound proteins were eluted in the same buffer containing 10 mM maltose and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

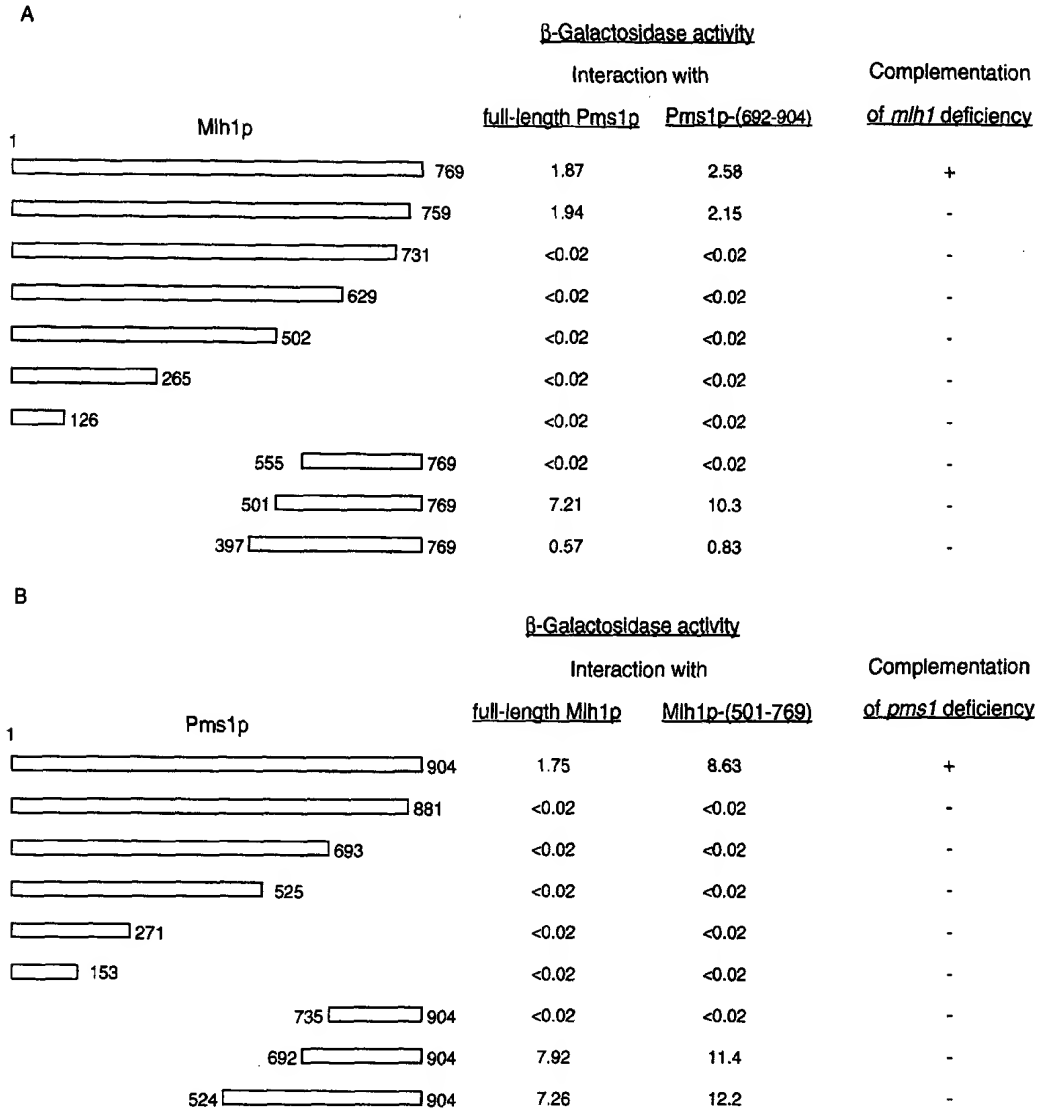

FIG. 1. Mapping of the interactive domains in Mlh1p and Pms1p. (A) Full-length (residues 1 to 769) or truncated Mlh1p was fused to the GAL4 activation domain, and the hybrid proteins were assayed in *S. cerevisiae* L40 for the ability to interact with either full-length Pms1p or the Mlh1p-interactive domain of Pms1p [Pms1p (692–904)] fused to the LexA DNA binding domain. The relevant Mlh1p sequences are illustrated diagramatically, with the amino acid residues of Mlh1p contained in each construct represented by the rectangles. Interaction was quantitated by β-galactosidase assays as described in Materials and Methods, and the values shown represent averages of the units of β-galactosidase activity from three independent assays. The variation in the relative β-galactosidase activities was within 10%. Complementation of *mlh1* deficiency was determined by a replica-plating analysis of the *hom3-10* reversion. For each construct, four patches were grown on selective medium in a 100- by 15-mm petri dish. +, 0 to 1 colonies of *hom3-10* revertants per patch; −, 30 to 50 colonies of *hom3-10* revertants per patch. (B) Schematic illustration of PMS1 derivatives fused to the GAL4 activation domain and their interaction with either full-length Mlh1p or the Pms1p-interactive domain of Mlh1p [Mlh1p(501–769)] fused to the LexA DNA binding domain. β-Galactosidase levels and complementation of *pms1* deficiency were determined as described for panel A; +, 0 to 1 colonies of *hom3-10* revertants, −, 30 to 50 colonies of *hom3-10* revertants.

Immunoblot analysis. The relative stabilities of the mutant Mlh1p and Pms1p proteins were assessed by Western blotting of yeast extracts. Cells with constructs encoding full-length Mlh1p and Pms1p and the truncated mutants Mlh1p(1–265) and Pms1p(1–271) were grown in 10 ml of minimal medium lacking tryptophan to a density of 2×10^7 cells/ml. Only these two truncated mutants were assayed for stability, because they demonstrated a different dominant negative effect in wild-type cells. Cells were pelleted, resuspended in 10 volumes of sample buffer (60 mM Tris [pH 6.8], 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% bromphenol blue), and broken by vortexing with an equal volume of glass beads (eight 15-s bursts). The lysate was boiled for 5 min, cleared by sonication for 15 s to shear DNA, and spun for 10 min at 12,000 rpm in a microcentrifuge. Ten microliters of this lysate was electrophoresed on a standard SDS–8% polyacrylamide gel and blotted to a nitrocellulose filter. The blot was preincubated in 6% nonfat dried milk prepared in TBS (20 mM Tris-HCl [pH 7.6] and 150 mM NaCl) and probed with a 1:400 dilution of monoclonal antibody 12CA5 (Boehringer Mannheim). The secondary antibody was a goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G antibody used at a 1:5,000 dilution (Pierce, Rockford, Ill.). The blot was visualized with chemiluminescent reagents from National Diagnostics (Atlanta, Ga.).

RESULTS

Identification of domains in Mlh1p and Pms1p sufficient for Mlh1p-Pms1p interaction. Evidence from yeast and human cells suggests that Mlh1p (hMLH1) and Pms1p (hPMS2) are likely to function as a heterodimer (30, 47). To investigate the

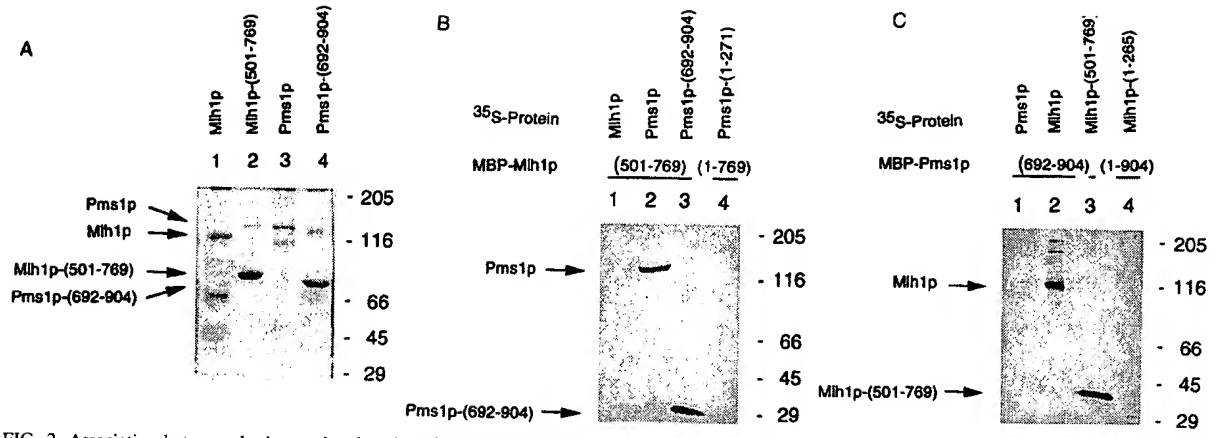

FIG. 2. Association between the interactive domains of Mlh1p and Pms1p in vitro. (A) MBP fusion proteins were prepared with amylose affinity columns as described in Materials and Methods, separated by SDS-PAGE (8% polyacrylamide), and stained with Coomassie blue. Lane 1, MBP-Mlh1p; lane 2, MBP-Mlh1p (501–769); lane 3, MBP-Pms1p; lane 4, MBP-Pms1p(692–904). Molecular size markers (in kDa) migrated as indicated. (B) Interaction of Mlh1p(501–769) with [^{35}S]methionine-labeled proteins. MBP fusion proteins were loaded onto amylose columns with [^{35}S]methionine-labeled proteins. Eluted proteins from these columns were resolved by SDS-PAGE (8% polyacrylamide) and analyzed by autoradiography. Lane 1, MBP-Mlh1p(501–769) plus [^{35}S]methionine-labeled full-length Mlh1p; lane 2, MBP-Mlh1p(501–769) plus [^{35}S]methionine-labeled full-length Pms1p; lane 3, MBP-Mlh1p(501–769) plus [^{35}S]methionine-labeled Pms1p(692–904); lane 4, MBP-Mlh1p plus [^{35}S]methionine-labeled Pms1p(1–271). (C) Interaction of Pms1p(692–904) with [^{35}S]methionine-labeled proteins. Lane 1, MBP-Pms1p(692–904) plus [^{35}S]methionine-labeled full-length Pms1p; lane 2, MBP-Pms1p(692–904) plus [^{35}S]methionine-labeled full-length Mlh1p; lane 3, MBP-Pms1p(692–904) plus [^{35}S]methionine-labeled Mlh1p(501–769); lane 4, MBP-Pms1p plus [^{35}S]methionine-labeled Mlh1p(1–265).

domains responsible for Mlh1p-Pms1p interaction, we constructed a series of yeast Mlh1p and Pms1p deletion mutants and tested the truncated proteins for interaction by using the yeast two-hybrid system. As shown in Fig. 1A, all but one carboxyl-terminal truncation of Mlh1p failed to interact with Pms1p. A carboxyl-terminal portion of Mlh1p corresponding to residues 501 to 769, Mlh1p(501–769), interacted with Pms1p, whereas Mlh1p(555–769) failed to bind to full-length Pms1p, suggesting that the Pms1p-interactive domain of Mlh1p lies in the carboxyl portion of Mlh1p. Interestingly, even though the final 13 amino acids are identical in the yeast and human MLH1 homologs (12, 44), this CTH motif of yeast Mlh1p apparently is not required for Pms1p interaction because deletion of the final eight amino acids of Mlh1p did not affect Mlh1p-Pms1p interaction as assayed in the two-hybrid system. We observed a reduced interaction of Mlh1p(397–769) with Pms1p, compared with the interactions observed for Mlh1p(501–769) and full-length Mlh1p. This weaker signal could be due to a reduced interaction or to either protein instability or abnormal folding. All *mlh1* deletion mutants showed a strong mutator phenotype as determined by a replica-plating assay of the *hom3-10* reversion (Fig. 1A).

Among Pms1p truncations (Fig. 1B), two carboxyl-terminal fragments, PMS1(692–904) and PMS1(524–904), showed strong interactions with full-length Mlh1p. In contrast, Pms1p (735–904) showed no interaction with Mlh1p. Again, by using the *hom3-10* reversion assay, each of the *pms1* deletion mutants were nonfunctional in repair. Further analysis showed that Mlh1p(501–769) interacted strongly with Pms1p(692–904) (see the third columns in Fig. 1A and B). The 10-fold higher apparent interaction relative to that observed for the full-length parent pair may result from an actual stronger interaction or from higher expression and/or increased stability of the truncated products. Taken together, the results of these two-hybrid assays suggest that domains within residues 501 to 769 of Mlh1p and residues 692 to 904 of Pms1p are primarily involved in Mlh1p-Pms1p interaction.

In vitro studies of the interactive domains. As a second means to define the domains sufficient for Mlh1p-Pms1p interaction, we tested the ability of Mlh1p(501–769) and Pms1p (692–904), expressed as stable fusion proteins with MBP in *E. coli* (Fig. 2A), to bind a series of [^{35}S]methionine-labeled proteins in a protein affinity chromatography assay. As indicated in Fig. 2B, [^{35}S]methionine-labeled full-length Pms1p and Pms1p(692–904) were retained specifically on the MBP-Mlh1p (501–769) column (lanes 2 and 3). Full-length Mlh1p did not interact with the Pms1p-interactive domain, Mlh1p(501–769) (lane 1), consistent with our previous observations (47). The amino-terminal Pms1p(1–271) (lane 4) showed no interaction with Mlh1p. As previously demonstrated (47), both full-length Pms1p and Pms1p(692–904) bound to a column containing full-length Mlh1p (data not shown).

The column with MBP-Pms1p(692–904) retained [^{35}S]methionine-labeled full-length Mlh1p and Mlh1p(501–769) (Fig. 2C, lanes 2 and 3) but not full-length Pms1p (lane 1). Consistent with the two-hybrid results, the full-length Pms1p column did not retain the amino-terminal Mlh1p(1–265) (lane 4). In a separate experiment, a full-length Pms1p column retained both full-length Mlh1p and Mlh1p(501–769) (data not shown). Neither Mlh1p(501–769) nor Pms1p(692–904) bound to a control column with MBP only (data not shown). These results are consistent with the two-hybrid studies presented above and strongly suggest that residues 501 to 769 of Mlh1p and residues 692 to 904 of Pms1p represent the primary domains necessary for Mlh1p-Pms1p interaction.

The effects of mutations in the MLH domain on mismatch repair function and Mlh1p-Pms1p interaction. Most known MutL homologs, including yeast Mlh1p and Pms1p, have significant homology (approximately 50% identical) in a stretch of 90 amino acids in the amino terminus (12, 44, 46) (Fig. 3A). The region, which we refer to as the MutL homology (MLH) domain, encompasses the MutL box sequence GFRGEAL, which is identical in most MutL homologs (Fig. 3A). To examine the structural and functional properties of the MLH domain, we made alanine substitutions at amino acid positions F96, R97, and G98 in the MutL box of yeast Mlh1p. This alanine mutagenesis strategy presumes that, while the functional group of the natural amino acid is removed, the alanine

A

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                                    ↓                                                    ↓↓↓
S. cerevisiae   MLH1   KEMMENSIDANATMIDILVKEGGIKVLQITDNGSGINKADLPILCERFTTSKLQKFEDLSQIQTYGFRGEALASISHVAR 109
                                    *
human           MLH1   KEMIENCLDAKSTSIQVIVKEGGLKLIQIQDNGTGIRKEDLDIVCERFTTSKLQSFEDLASISTYGFRGEALASISHVAH 112
S. cerevisiae   PMS1   KELVDNSIDANANQIEIIFKDYGLESIECSDNGDGIDPSNYEFLALKHYTSKIAKFQDVAKVQTLGFRGEALSSLCGIAK 139
human           PMS2   KELVENSLDAGATNIDLKLKDYGVDLIEVSDNGCGVEEENFEGLTLKHHTSKIQEFADLPQVETFGFRGEALSSLCALSD 119
human           PMS1   KELIENSLDAGATSVDVKLENYGFDKIEVRDNGEGIKAVDAPVMAMKYYTSKINSHEDLENLTTYGFRGEALGSICCIAE 105
E. coli         MutL   KELVENSLDAGATRIDIDIERGGAKLIRIRDNGCGIKKDELALALARHATSKIASLDDLEAIISLGFRGEALASISSVSR 107
S. typhimurium  MutL   KELVENSLDAGATRVDIDIERGGAKLIRIRDNGCGIKKEELALALARHATSKIASLDDLEAIISLGFRGEALASISSVSR 107
S. pneumoniae   HexB   KELVENAIDAGSSQIIIEIEEAGLKKVQITDNGHGIAHDEVELALRRHATSKIKNQADLFRIRTLGFRGEALPSIASVSV 108
                      ••••••••••••• •••   • • • • ••• ••        •• ••••   •• • • ••••••• •• •
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B

Protein	Relative mutation rate	β-galactosidase activity Interaction with Pms1p
WT Mlh1p	1	1.87
Mlh1pΔ	1158	ND
WT Pms1p	1	1.75
Pms1pΔ	1636	ND
Mlh1p-F96A	798	1.33
Mlh1p-R97A	256	1.64
Mlh1p-G98A	693	1.72
Mlh1p-G98V	799	<0.02
Pms1p-F126A	639	1.68
Mlh1p-A41S	1	1.92
Mlh1p-A41F	1620	<0.02
Mlh1p-A41G	61	0.42

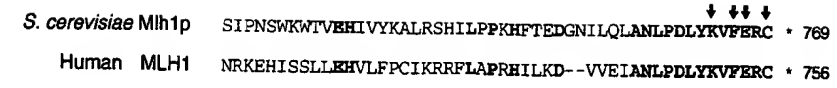
FIG. 3. Effects of mutations in the MLH domain on DMR function and Mlh1p-Pms1p interaction. (A) Sequence comparisons of the MLH domains of the MutL homologs *S. cerevisiae* Mlh1p (46), hMLH1 (12), *S. cerevisiae* Pms1p (27), hPMS2 (42), human PMS1 (42), *E. coli* MutL (31), *Salmonella typhimurium* MutL (35), and *Streptococcus pneumoniae* HexB (48). Amino acids that are identical for at least five proteins are marked with a dot. The arrowheads indicate the positions at which amino acid changes were made. The asterisk denotes the predicted amino acid change S44F in the hMLH1 HNPCC mutation (12). (B) DMR function and interaction of wild-type (WT) and mutant proteins. Mutation rates were determined by the fluctuation assay as described in Materials and Methods and are presented relative to the rate observed for WT Mlh1p (9.5×10^{-9}) or WT Pms1p (1.1×10^{-8}). The strains used to determine the null mutation rates of Mlh1Δ and Pms1pΔ have been described previously (46). ND, not determined. Interaction was quantitated as described in the legend to Fig. 1.

substitutions do not perturb the overall structure or stability of the protein (13). As shown in Fig. 3B, alanine substitutions at F96, R97, and G98 resulted in loss of mismatch repair function as determined by the inability to complement a yeast *mlh1*Δ mutation but did not affect interaction with Pms1p. However, substitution of glycine at residue 98 with the larger and more hydrophobic valine not only caused a strong mutator phenotype but also abolished binding to Pms1p. We also demonstrated that a Pms1p mutant with a point mutation in the MutL box, Pms1p-F126A (analogous to Mlh1p-F96A), also showed a strong mutator phenotype but retained interaction with Mlh1p.

Previous studies showed that an S44F substitution in the MLH domain of hMLH1 was a likely cause of HNPCC (12). To test the effect of an analogous mutation in yeast, we introduced point mutations at position A41 of yeast Mlh1p. At position 41, yeast Mlh1p contains alanine whereas hMLH1 contains serine (12) (Fig. 3A). As shown in Fig. 3B, the replacement of the alanine with serine in yeast Mlh1p did not affect either repair function or Mlh1p-Pms1p interaction. In contrast, substitution with phenylalanine at position 41 in yeast Mlh1p resulted in loss of both mismatch repair function, equivalent to a null mutation, and interaction with Pms1p. Interestingly, the replacement of A41 with a structurally similar glycine had only a minor effect on both repair function and binding of Pms1p. These results support the finding that the hMLH1 mutation S44F is causal in HNPCC families carrying this mutation (see Discussion).

Mutations in the CTH motif of Mlh1p cause loss of DMR function but do not affect interaction with Pms1p. The final 13 amino acids of the yeast Mlh1p and the hMLH1 proteins are identical (12, 44) (Fig. 4A). The deletion studies described above suggest that this portion of yeast Mlh1p is not involved in Mlh1p-Pms1p interaction (Fig. 1A). However, this highly conserved motif is required for mismatch repair function because deletion of the final eight amino acids [Mlh1p(1–761)] resulted in a strong mutator phenotype (Fig. 1A). To identify important residues responsible for mismatch repair function, we generated missense mutations in the CTH motif of the yeast Mlh1p protein. These missense mutations were tested for complementation of a yeast *mlh1*Δ strain and interaction with Pms1p. As shown (Fig. 4B), these substitutions had no effect on interaction with Pms1p, consistent with our deletion analysis (Fig. 1A). By contrast, most substitutions at positions K764, F766, E767, and C769 resulted in repair-deficient alleles. While the conservative substitution K764R retained mismatch repair activity, K764E and F766A eliminated function. Surprisingly, the conservative change E767D resulted in loss of mismatch repair function. Possibly, E767 is involved in amino acid-amino acid contact that the slightly smaller aspartic acid cannot accommodate. These results further confirm that the CTH motif of Mlh1p is essential for DMR but not for Mlh1p-Pms1p interaction and indicate that Mlh1p-Pms1p interaction is necessary but not sufficient for mismatch repair function.

A



B

Protein	Relative mutation rate	β-galactosidase activity Interaction with Pms1p
WT Mlh1p	1	1.87
Mlh1pΔ	1158	ND
K764E	699	1.93
K764R	2	1.82
F766A	968	2.14
E767D	603	1.76
C769A	3	1.91
C769S	289	1.88

FIG. 4. The CTH motif of Mlh1p is necessary for DMR function but not Mlh1p-Pms1p interaction. (A) An alignment between the carboxyl-terminal parts of the yeast Mlh1 and the hMLH1 proteins. Identical amino acids are shown in boldface. The arrowheads indicate the positions at which amino acid changes were made. Asterisks denote the end of each protein. (B) DMR function and interaction of wild-type and mutant proteins. Mutation rates were determined by the fluctuation assay as described in Materials and Methods and are presented relative to the rate observed for the wild-type (WT) Mlh1p, which is 9.5×10^{-9}. The strain used to determine the null mutation rate of Mlh1pΔ has been described previously (46). ND, not determined. Interaction was quantitated as described in the legend to Fig. 1.

Certain *MLH1* and *PMS1* mutations show dominant negative effects. As described above, mutational analysis of the yeast Mlh1p and Pms1p proteins revealed two elements of unknown function, the MutL box and the CTH motif, both of which are required for DMR but are apparently not involved in Mlh1p-Pms1p complex formation. The presence of excess protein altered in these functional domains could block DMR in a wild-type cell due to either the formation of a nonfunctional repair complex or the inability to interact with other components. We overexpressed several altered Mlh1p proteins, constructed in a yeast expression vector under an ADH promoter, in a wild-type strain and tested for mutator activity. Altered Mlh1p and Pms1p constructs containing the Pms1p-interaction domain of Mlh1p [Mlh1p(501–769)] and the Mlh1p-interaction domain of Pms1p [Pms1p(692–904)] caused mutator phenotypes in wild-type cells (Table 1). Construct Mlh1p(1–761), which was deleted for the CTH motif, and the MutL box mutants Mlh1p-F96A and Pms1p-F126A also exhibited a dominant negative mutator effect in wild-type cells.

We also overexpressed Mlh1p and Pms1p sequences that do not show interaction with full-length Pms1p and Mlh1p. Overexpression of Mlh1p(1–265) and Mlh1p(734–769) exerted a dominant negative effect on mismatch repair (Table 1). Interestingly, Mlh1p-K619del overexpression, the equivalent of an HNPCC mutation associated with DMR deficiency in phenotypically normal human cells, also resulted in a dominant negative effect. The dominant negative effects resulting from Mlh1p (1–265), Mlh1p(734–769), and Mlh1p-K619del overexpression are apparently not mediated by interaction with PMS1.

In contrast to the effect observed with Mlh1p(1–265), overexpression of the Pms1p(1–271) fragment did not result in a dominant negative mutator effect. To determine whether this might be due to inappropriate expression or reduced stability of the Pms1p construct, full-length Mlh1p and Pms1p and the carboxyl-terminal truncations Mlh1p(1–265) and Pms1p(1–271) were tagged with an epitope corresponding to a nine amino-acid region of the HA protein of influenza virus (17). The tagged proteins were overexpressed in the wild-type cell and probed with a monoclonal HA antibody to determine the relative levels of the proteins. As indicated in Fig. 5, both Mlh1p(1–265) (lane 3) and Pms1p(1–271) (lane 5) were expressed at higher levels than full-length Mlh1p (lane 2) and Pms1p (lane 4). An additional lower band was observed in the Pms1p(1–271) sample, suggesting that the truncated protein was unstable in the cell. Thus, the relative instability of Pms1p (1–271) may account for the lack of a dominant negative effect.

DISCUSSION

Although the MutL protein is an essential component of the methyl-directed mismatch repair system in *E. coli*, little is known about MutL function. No enzymatic function or DNA mismatch specific binding activity has been reported for MutL. It has been proposed that MutL functions as a "molecular matchmaker," presumably acting to stabilize the MutS-mismatch-DNA complex (51). Our previous studies suggested that in the yeast *S. cerevisiae* the MutL equivalent, a Mlh1p-Pms1p

TABLE 1. Dominant negative effects of certain Mlh1p and Pms1p constructs[a]

Construct	Mutation rate in WT cells[b]	Interaction with Pms1p or Mlh1p[c]
Vector only	1.39×10^{-8} (1)	−
WT Mlh1p	7.66×10^{-9} (0.6)	+
WT Pms1p	1.06×10^{-8} (0.8)	+
Mlh1p(501–769)	2.12×10^{-6} (152)	+
Pms1p(692–904)	1.86×10^{-6} (134)	+
Mlh1p-K619del	6.19×10^{-7} (44.5)	−
Mlh1p(1–265)	3.98×10^{-7} (28.6)	−
Pms1p(1–271)	9.95×10^{-9} (0.7)	−
Mlh1p-F96A	7.85×10^{-6} (565)	+
Pms1p-F126A	3.57×10^{-6} (256)	+
Mlh1p(1–761)	3.33×10^{-6} (240)	+
Mlh1p(734–769)	4.56×10^{-7} (32.8)	−

[a] Full-length or amino-terminal portions of the *MLH1* and *PMS1* cDNA sequences were subcloned in pCoB-5006; fragments encoding the carboxyl-terminal portions of both proteins were fused in frame with the ATG start codon in pJAS (see Materials and Methods). WT, wild type.

[b] The mutation rate relative to that of the vector-only construct is shown in parentheses.

[c] Interaction was determined by the two-hybrid assay; + and − indicate interaction and no interaction, respectively.

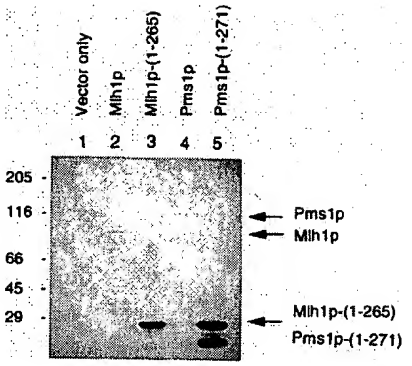


FIG. 5. Western blot analysis of HA-tagged proteins. Cell lysates were prepared from yeast cells containing full-length or truncated proteins tagged with an HA epitope and immunoblotted with monoclonal antibody 12CA5 as described in Materials and Methods. Lane 1, pCoB-5006 vector only; lane 2, full-length Mlh1p; lane 3, Mlh1p(1–265); lane 4, full-length Pms1p; lane 5, Pms1p(1–271). Note that lane 5 shows two products: the upper band corresponds to the expected Pms1p(1–271), and the lower band corresponds to a further truncated product. Molecular size markers (in kDa) migrated as indicated.

heterodimer, interacts with Msh2p bound to mismatch-containing DNA (47). In humans, MLH1 and PMS2 can be copurified as a heterodimer that complements the DMR defect in nuclear extracts of the colorectal tumor cell line defective in MLH1 (30). Through the use of the yeast two-hybrid system and of in vitro affinity protein chromatography, we have identified domains that appear to be primarily responsible for mediating the interaction between the yeast DMR proteins Mlh1p and Pms1p. Residues 501 to 769 of Mlh1p and residues 692 to 904 of Pms1p were shown to be required for Mlh1p-Pms1p interaction. *MLH1* and *PMS1* mutants in which these domains have been deleted failed to interact and were unable to complement *mlh1*- or *pms1*-deficient cells. Additionally, using site-directed mutagenesis, we have identified two highly conserved regions, namely the MutL box of both proteins and the CTH motif of Mlh1p, which are each necessary for DMR function but which are not required for Mlh1p-Pms1p interaction. We have also shown that certain Mlh1p and Pms1p mutants, with or without the interactive domains, exhibited dominant negative effects when overexpressed in wild-type cells.

The functional domains of the Mlh1p and Pms1p proteins as defined in this report are summarized in Fig. 6. Our results indicate that the carboxyl-terminal 33% of Mlh1p (not including the last 13 amino acids) and the carboxyl-terminal 23% of Pms1p are sufficient for Mlh1p-Pms1p interaction. Deletion of the interactive domains resulted in loss of both DMR function and Mlh1p-Pms1p interaction. However, deletion constructs containing only the Pms1p-interactive domain [Mlh1p(501–769)] or the Mlh1p-interactive domain [Pms1p(692–904)] retained Mlh1p-Pms1p dimerization activity but abolished DMR function. Therefore, Mlh1p-Pms1p interaction is necessary but not sufficient for DMR. There is no amino acid sequence homology between the two interactive domains of Mlh1p and Pms1p. However, the interacting regions of yeast Mlh1p and Pms1 have homology with the corresponding regions of their human counterparts, MLH1 and PMS2. We suggest that these homologous regions are responsible for interactions of the human proteins. In addition, during a two-hybrid search for Mlh1p-interacting proteins, we detected a protein with strong homology to Pms1p (51a). The carboxyl-terminal portion of this protein, which we refer to as Mlh3p, shows striking similarity to the final 200 amino acids of Pms1 corresponding to the Mlh1p-interactive domain [Pms1p(692–904)]. These similarities are restricted to three subdomains with 25, 10, and 11 residues separated by about 100 and 20 residues, respectively (Fig. 7). Because these conserved subdomains are likely to comprise the elements necessary for binding of Pms1p and Mlh3p to Mlh1p, further study of these subdomains should be informative.

Results of two-hybrid assays and in vitro protein affinity chromatography indicate that Mlh1p-Pms1p interaction does not require amino-terminal portions of either protein. Our results do not exclude the possibility that the amino-terminal regions of Mlh1p and Pms1p play a role in maintaining a proper protein conformation necessary for the interaction. Two mutants with point mutations in the MLH domain of Mlh1p, Mlh1p-A41F and Mlh1p-G98V, disrupt Mlh1p-Pms1p interaction (Fig. 3B). Previously, it was suggested that alteration of hMLH1 by an S44F substitution, observed as the result of a germline mutation in an HNPCC family, was predicted to alter the hMLH1 protein conformation (12). Residue A41 of yeast Mlh1p, analogous to residue S44 of hMLH1, is in a highly conserved region of the MutL homologs (Fig. 3A). Chou-Fasman predictions suggest that both A41F and G98V substitutions change the predicted secondary structure in these regions and could therefore affect the overall conformation of Mlh1p. Alternatively, some regions in the amino-terminal domains of Mlh1p and Pms1p may have a role in heterodimer stabilization between full-length proteins in vivo.

An important implication of this study is that the highly conserved MLH domain of both proteins and the CTH motif of Mlh1p do not appear to be essential for Mlh1p-Pms1p interaction but are critical for DMR function. Figure 6 shows the region of highest conservation between Mlh1p and Pms1p, the MLH domain which encompasses the consensus MutL box sequence GFRGEAL, and a region with the final 13 amino acids identical between the yeast Mlh1p and the hMLH1 proteins (CTH motif). Most alterations in the MutL box and the CTH motif eliminated DMR function but not Mlh1p-Pms1p interaction, suggesting that these two functional domains have an intrinsic DMR activity and/or mediate interactions with other proteins in the DMR pathway. Relevant to the possibility

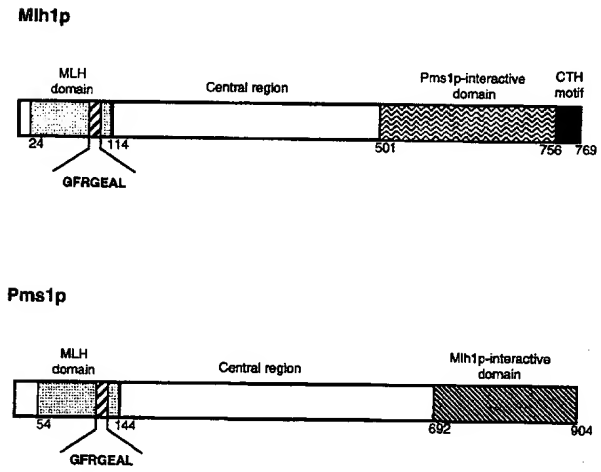


FIG. 6. Schematic structures of Mlh1p and Pms1p. The conserved amino-terminal MLH domain consists of 90 residues in each protein and encompasses the MutL box sequence GFRGEAL. The CTH motif in Mlh1p contains the final 13 amino acids that are identical between the yeast Mlh1 and the hMLH1 proteins.

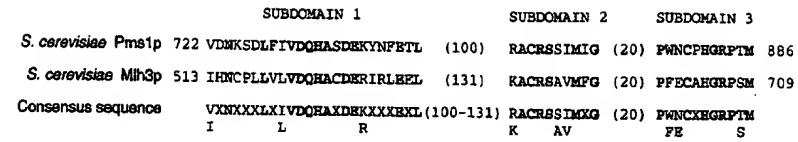


FIG. 7. Subdomains conserved between the Mlh1p-interactive domain of Pms1p [Pms1p(692–904)] and the carboxyl-terminal portion of Mlh3p (accession number Z73520). Bold letters correspond to identical amino acids. X, nonconserved residue. The number of residues between the different subdomains is indicated in parentheses.

of a direct mismatch repair activity for MutL proteins, a novel class of ATP binding and hydrolysis domains appears to be conserved among the B subunit of the type II topoisomerases, *E. coli* gyrase, and the MutL protein family (9). Both *E. coli* gyrase and the type II topoisomerase subunit B can bind an ATP analog (9, 24). The three putative ATP-binding motifs are present in the MLH domain of Mlh1p and Pms1p (9) (Fig. 3). Therefore, the Mlh1p-Pms1p heterodimer may cooperate with Msh proteins in providing the ATP binding and hydrolysis functions necessary for mismatch correction.

The dominant negative effect of overexpression of the MutL box mutants Mlh1p-F96A and Pms1p-F126A and the CTH motif deletion Mlh1p(1–761) may result from mutant proteins that form nonfunctional Mlh1p-Pms1p dimers that either fail to interact appropriately with other proteins or cannot carry out a biochemical function, e.g., ATP binding and hydrolysis. In contrast, the dominant negative effects of Mlh1p(1–265), Mlh1p-K619del, and Mlh1p(734–769), all of which are unable to bind Pms1p, are likely to occur through a Pms1p-independent mechanism. These dominant negative effects may result from competitive titration of other critical components, e.g., proteins of the repair complex. The dominant negative mutator effect of Mlh1p-K619del is analogous to the effect of an hMLH1 mutant, i.e., hmlh1-K618del, that appears to compromise DMR activity in normal cells of HNPCC patients (45). We stress that the dominant negative effects in the present study appear to require overexpression of the mutant proteins (Fig. 5). Therefore, in the case of the potential hMLH1 dominant negative mutation (45), we would predict that the mutant allele is expressed at a level higher than the level of expression of the wild-type allele. Supporting this argument is the recent observation that a human *PMS2* nonsense mutation, *hPMS2-134* (45), that appears to be a dominant negative germline mutation is expressed at a significantly higher level than the level of expression of the wild-type allele in cells from clinically affected patients (41a).

Recently, Aronshtam and Marinus (5) have reported the use of dominant negative mutations to identify functional domains of *E. coli* MutL protein. These dominant negative effects also required high-level expression of the mutant proteins. Most MutL dominant negative mutations were found to be located in the conserved amino-terminal portion of the MutL protein (5), a finding that supports our observations with yeast. Furthermore, multicopy plasmids containing the wild-type *mutS* or *mutH* gene could suppress the dominant negative mutations in the *mutL* gene encoding the carboxyl-terminal but not the amino-terminal part of the protein, suggesting that the two parts of the MutL protein might perform different functions (5). It will be of interest to ask whether overexpression of certain genes (e.g., *MSH2* and *MSH6*) can overcome the dominant negative effect of the *MLH1* and *PMS1* mutations.

Finally, recent work suggests that an early step in DMR in both yeast and human requires the replication processivity protein PCNA (54). In yeast, Pcnap was shown to interact with both Mlh1p and Msh2p, suggesting a linkage between the Mlhp and Mshp heterodimers. However, Mlh1p mutants with point mutations in the MutL box no longer interact with Pcnap (51a), although the Mlh1p-Pms1p interaction is preserved, suggesting that the MutL box of Mlh1p may be involved in the Mlh1p-Pcnap interaction and thus in the formation of a higher-order DMR complex. In summary, our results support a model in which one of the functions of the Mlh1p-Pms1p heterodimer is to recruit other components to the mismatch repair complex. Like Mlh1p-Pms1p interaction, the putative interaction of the Mlh1p-Pms1p heterodimer with other proteins is presumably necessary for the DMR function. The results of the domain mapping of Mlh1p and Pms1p presented here should aid in the identification of new components in the DMR process and in the evaluation of the functional and disease significance of mutations found in the human MutL homologs in cancer families.

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